Supplementary Data

Materials and Methods

Cell culture

Human embryonic stem cells (hESCs) (H9; Wicell Research Institute, Madison, WI) and previously reported human induced pluripotent stem cells (hiPSCs) (SES8) were cultured on mitomycin-treated mouse embryonic fibroblasts in a typical hESC medium.[1] Human vascular smooth muscle cells (HVSMCs; ScienCell Research Laboratories, Carlsbad, CA) and human umbilical vein endothelial cells (HUVECs; ScienCell Research Laboratories) were cultured in the SMC growth medium (ScienCell Research Laboratories) and the endothelial growth medium (EGM)-2 (Lonza, Walkersville, MD), respectively.

Vascular differentiation of hESCs and hiPSCs

After feeder-free culture for 2 days on Matrigel (BD Biosciences, Bedford, MA), hESCs and hiPSCs were dissociated to generate embryonic bodies (EBs). The EBs were cultured in differentiation medium I containing 10% knockout serum replacement (20%; Gibco, Grand Island, NY), 10 ng/mL vascular endothelial growth factor (VEGF; R&D Systems, Minneapolis, MN), 10 ng/mL bone morphogenetic protein (BMP)-4 (ProSpec, Rehovot, Israel), 10 ng/mL basic fibroblast growth factor (bFGF; ProSpec), and 3 ng/mL Activin A (R&D Systems) in the DMEM/F12, under hypoxic conditions (3% O_2).[2,3] After 8 days of culture, EBs were attached to a gelatin-coated dish and cultured for another 7 days in differentiation medium II containing 50 ng/mL VEGF, 20 ng/mL BMP-4, and 5 ng/mL bFGF in the DMEM/F12, under normoxic conditions. On day 15 of differentiation, the EBs were dissociated into single cells and labeled with phycoerythrin-conjugated anti-human CD34 IgGs (Dako, Inc., Carpinteria, CA). Controls were stained with the appropriate isotype-matched nonspecific IgGs. CD34-positive cells were purified with a FACSAria III cell sorter using CellQuest acquisition software (BD Biosciences). Sorted CD34-positive cells were further differentiated into ECs or SMCs by culturing in the EGM-2 supplemented with 50 ng/mL VEGF and 10 nM SB431542 (Calbiochem, San Diego, CA), or the SMC growth medium supplemented with 50 ng/mL platelet-derived growth factor-BB (R&D Systems) and 10 ng/mL transforming growth factor-β (R&D Systems), respectively.[4,5]

Reverse transcription–polymerase chain reaction

Total RNA was extracted from cells with the Trizol reagent (Invitrogen, Carlsbad, CA). The cDNA was synthesized using the Superscript first-strand synthesis system (Invitrogen) and amplified through 25–35 polymerase chain reaction (PCR) cycles with gene-specific primers. Primer sequences are listed in Supplementary Table S1.

Immunofluorescence staining

Cells were fixed and blocked with 10% normal goat serum, and incubated with primary IgGs against CD34 (Dako, Inc.), CD31 (Dako, Inc.), vascular endothelial-cadherin (VE-Cad; R&D Systems), SM22α (Abcam, Cambridge, MA), α-smooth muscle actin (α-SMA; R&D Systems), or with irrelevant nonspecific IgGs. Cells were incubated with fluorescence-conjugated secondary IgGs (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and imaged with a fluorescence microscope (Nikon, Melville, NY). Nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI). The endothelial phenotype of differentiated cells was confirmed by staining with DiI-labeled acetylated LDL (acLDL; Molecular Probes, Eugene, OR) and FITC-conjugated *Ulex europaeus* agglutinin (ulex-lectin; Sigma, St. Louis, MO).

Flow cytometry

Cells pretreated with human-FcR blocking reagent (Miltenyi Biotec, Gladbach, Germany) were labeled with fluorescence-conjugated primary IgGs against CD34, CD31, VE-Cad, or α-SMA for 2 h at 4°C and washed with an ice-cold buffer. Samples were analyzed with controls stained with the appropriate isotype-matched nonspecific IgGs using a FACS Accuri flow cytometer (BD Biosciences).

Tube formation assay

A total of 3×10^4 cells were seeded into each well of a Matrigel-coated 24-well plate at ratios of 100:0, 60:40, and 40:60 (DiI-labeled ECs: DiO-labeled SMCs). After overnight incubation in EGM-2, tubule networks were quantified by measuring the tubule area in four random microscope fields.

Angiogenesis protein array

The Human Angiogenesis Proteome Profiler™ array kit (R&D Systems) was used to determine the level of angiogenesis-related proteins secreted from ECs and SMCs. A conditioned medium (CM) was prepared by culturing cells in serum-free basal media at 37°C for 24 h. The CM was cleared out by centrifugation with a Microcon filter (molecular weight cutoff = 10 K, Amicon Division, Danvers, MA), and then incubated with the antibody cocktail. After blocking the nitrocellulose membrane, the sample and detection antibody cocktail mixture was added to the membranes and incubated overnight at 4°C. The membranes were washed, incubated with Streptavidin-HRP, and subsequently visualized with Chemi Reagent Mix. The array data were quantified by densitometry using the NIH ImageJ analyzer.

Chemotactic migration assay

The chemotactic migration assay was performed with a modified Boyden chamber (Costar, Cambridge, MA), in which, the lower side of the filter membrane was coated with 0.5 mg/mL of type I collagen (Sigma). The CM harvested from ECs was added to the lower chamber and 1×10^4 of SMCs were placed in the upper chamber. After incubation for 24 h at 37°C, the membrane was stained with hematoxylin and eosin (H&E). Migrated cells that attached to the lower side of the filter were counted.

Supplementary Table S1. Primer List for RT-PCR

Primer	*Forward (5′ to 3′)*	*Reverse (5′ to 3′)*
Exo Oct4	CGAATTCGACCCAAGTTTGT	CCTTGGAAGCTTAGCCAGGT
Exo Sox2	CGAATTCGACCCAAGTTTGT	GGGCTGTTTTTCTGGTTGC
Exo Nanog	CGAATTCGACCCAAGTTTGT	AGTCTCCGTGTGAGGCATCT
Endo Oct4	GACAGGGGGAGGGGAGGAGCTAGG	CTTCCCTCCAACCAGTTGCCCCAAAC
Endo Sox2	GGGAAATGGGAGGGGTGCAAAAGAGG	TTGCGTGAGTGTGGATGGGATTGGTG
CD31	AGGTGTTGGTGGAAGGAGTG	CGTGTAGTTGCCACTGTGCT
KDR	ATCCTGGGGTAAAGATTGAT	TTAGCTTGCAAGAGATTTCC
VE-Cad	CGGTCAAACTGCCCATACTT	CAGCCCAAAGTGTGTGAGAA
CD34	TTGGCCAAGACCAGCAGTAG	CTCCAGCTGTGCGGAGTTTA
Tie2	CTGCCTAAAA GTCAGACCAC	GTGTTGACTCTAGCTCGGAC
GAPDH	GGAGATTGTTGCCATCAACG	GTGATGGCATGGACTGTGGT

Wound analysis

On days 0, 3, 7, and 10 after treatment, the wounds were documented using a digital camera. Briefly, the open wound area was measured by tracing the wound margin on the wound image. The pixel area was calculated and expressed as a percentage of the pixel area of the original wound at day 0. To measure the granulation tissue area, wound tissues were harvested on day 10 after treatment and longitudinally cut in half through the least-healed portion. They were fixed, embedded in paraffin, and serially sectioned perpendicular to the wound surface to analyze the granulation tissue area and the immunohistology. Wound sections with the largest epithelial gap (maximal distance between the epithelial edges; considered to be a central region of the wound) were stained with H&E. The granulation tissue area was analyzed on stained digitalized images with the NIH ImageJ analyzer by tracing the wound boundary that was considered as the presence of intact hair follicles and the transition from normal to hypertrophic epidermis, and calculating the pixel area. The number of sections examined for the following experiments ranged from 8 to 10 per wound. The number of sections examined for the following experiments ranged from 8 to 10 per wound.

Quantification of perfused blood vessel area and immunohistochemistry

To assess the perfused blood vessel area, serial tissue sections of Matrigel/collagen harvested from Balb/c nude mice were stained with H&E. The perfused vessel area in stained digitalized images was analyzed with the NIH ImageJ analyzer by counting the pixels of the erythrocyte-filled vessel area. This area was expressed as a percentage of the total tissue area on the image. To measure the capillary and arteriole densities, the sections of Matrigel/collagen and dermal wound tissues were blocked and stained with species–nonspecific primary IgGs against CD31 (Abcam) or α-SMA (Abcam), respectively. The sections were incubated with fluorescence-conjugated secondary IgGs, and nuclei were stained with DAPI. To detect the presence of transplanted human cells in the mouse vasculature, sections were costained with anti-human leukocyte antigen (HLA) primary IgGs (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunofluorescence images were taken with a fluorescence microscope (Nikon). The number of sections examined ranged from 6 to 10 per wound.

References

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